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A new willow leaf blotch miner of the genus *Phyllocnistis* (Lepidoptera: Gracillariidae: Phyllocnistinae) from Japan, with pupal morphology and genetic comparison of Salicaceae mining species using DNA barcodes

Shigeki KOBAYASHI[1), 2)], Yoshiko SAKAMOTO[2)], Utsugi JINBO[3)], Akihiro NAKAMURA[4)], and Toshiya HIROWATARI[2)]

[1)] Research Fellow of the Japan Society for the Promotion of Science

[2)] Entomological laboratory, Graduate School of life & Environmental Sciences, Osaka Prefecture University, Sakai, Osaka, 599-8531 Japan

[3)] Ito Laboratory, Department of General Systems Studies, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro, Tokyo, 153-8902 Japan

[4)] Laboratory of Landscape Architecture and Conservation, Graduate School of life & Environmental Sciences, Osaka Prefecture University, Sakai, Osaka, 599-8531 Japan

Abstract A new willow leaf blotch miner, *Phyllocnistis gracilistylella* sp. nov. (host plants: *Salix gracilistyla*, *S. serissaefolia*, *S. integra*, and *S. gilgiana*) is described and compared with *P. unipunctella* (Stephens, 1834) and *P. saligna* (Zeller, 1839). *Phyllocnistis unipunctella* is newly recorded from the mainland Japan. Adult morphologies and life histories of the three Salicaceae mining species are described with photographs and illustrations. The pupal morphologies of the new species and *P. saligna* are described with the use of a scanning electron microscope. The new species and *P. saligna* occurred together, but differed in the position of the mines; the former utilized only the leaf, mostly the lower surface, while the latter utilized the stem and upper surface of the leaf and partly the leaf edge at the cocoon stage. The new species was clearly divided from *P. saligna* not only by adult and pupal morphologies but also by DNA barcodes. Molecular analysis using reference DNA barcods also indicated that Japanese representatives of *P. saligna* are more closely related to *P. ramulicola* than to European *P. saligna*.

Key words *Phyllocnistis saligna*, *Salix*, *Populus*, taxonomy, pupa, DNA barcoding.

Introduction

Adult Phyllocnistinae as a group are among the smallest microlepidopteran leaf-miners, with a wing expanse of only 4-8 mm, similar in appearance to each other, with a silvery vestiture. This subfamily is characteristic in having a smooth head, antennae with a long and heavy, sometimes small, basal eye-cap, the male abdominal segment 8 membranous, and coremata usually present (Kuroko, 1982; Davis and Robinson, 1998). For more than a century, the placement of the largely cosmopolitan genus *Phyllocnistis* has vacillated between several families (Heinemann and Wocke, 1870), and recently it has been treated as a subfamily of the Gracillariidae (Davis, 1987; Common, 1990).

Approximately 90 species are known globally; nearly 26 plant families have been reported as hosts (De Prins and De Prins, 2005, 2010; Kawahara *et al.*, 2009). The genus has been generally poorly studied because of its small size and the difficulty of identifying species. Morphological characters of the larval stage poorly define species of *Phyllocnistis*, however, Kawahara *et al.* (2009) demonstrated that pupal morphology provides the most informative characters for distinguishing species in the genus.

Molecular data, such as DNA sequences, have been increasingly used for identifying morphologically closely related species. One of the more remarkable approaches is DNA barcoding, a technique for identification of organisms based on a short and standardized fragment of DNA sequences, termed the "DNA barcode" (648 base pairs of the mitochondrial cytochrome oxidase subunit I (COI) for animals). Since the concept of DNA barcodes was proposed by Hebert *et al.* (2003), this methodology has attracted the attention of researchers. Currently a global project, the international Barcode of Life, is in progress to create a comprehensive reference barcode database (http://ibol.org/). DNA barcoding has been applied for various entomological studies as an identification tool (Jinbo *et al.*, 2011). The Lepidoptera is one of the principal target groups for DNA barcoding and a global Lepidoptera barcoding project has been launched (Lepidoptera Barcode of Life, http://www.lepbarcoding.org/). Currently, DNA barcode sequences of 50,000 lepidopteran species, including Gracillariidae, are available through the official DNA barcode database (Barcode Life Data Systems, BOLD, Ratnasingham and Hebert, 2007; Lepidoptera Barcode of Life: http://www.lepbarcoding.org/). This library enables us to compare genetic differences among closely related

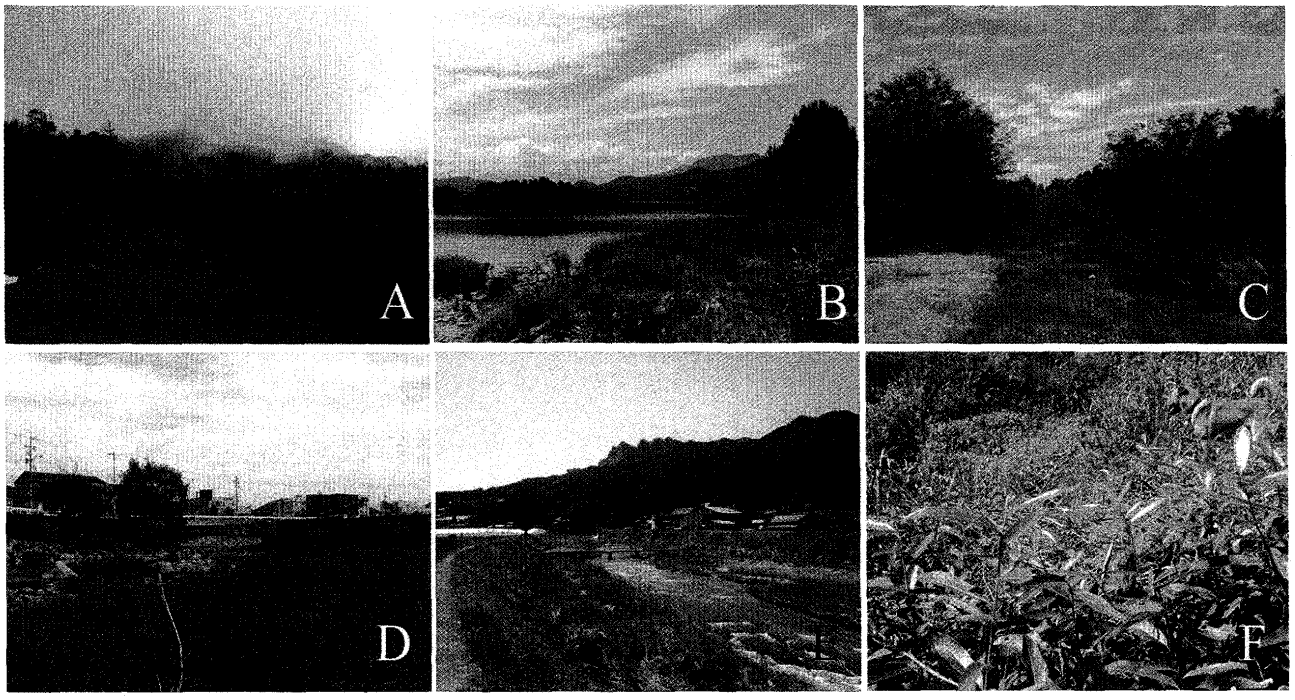

Fig. 1. Habitats and larval host plants of *Phyllocnistis* species.
A: Mt. Kasaga-dake, Takayama, Nagano Pref., 1600 m. B: Sai River, Nagano, Nagano Pref., 350 m. C: Azusa River, Matsumoto, Nagano Pref., 300 m. D: Nabari River, Natsumi, Nabari, Mie Pref., 200 m. E: Type locality of *P. gracilistylella* sp. nov., Imai, Soni, Nara Pref., 400 m. F: Young stem shoots and leaves of *Salix gracilistyla* in the type locality.

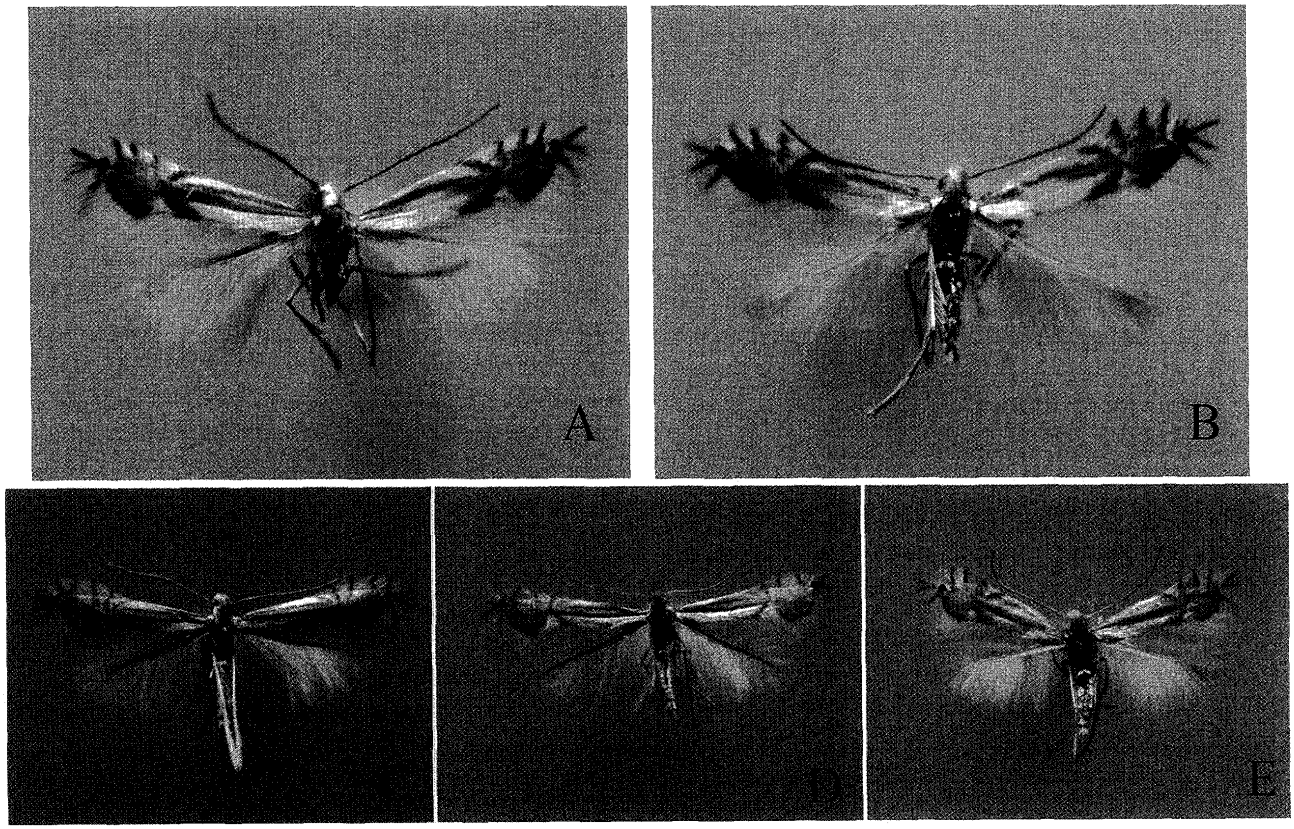

Fig. 2. Adults of Salicaceae mining *Phyllocnistis* species from Japan. A: *P. gracilistylella* sp. nov., holotype ♂. B: *Ditto*, paratype, ♀. C: *P. saligna* mining stem of *Salix gracilistyla*. D: *P. saligna* mining leaf of *Populus sieboldii*. E: *P. unipunctella*.

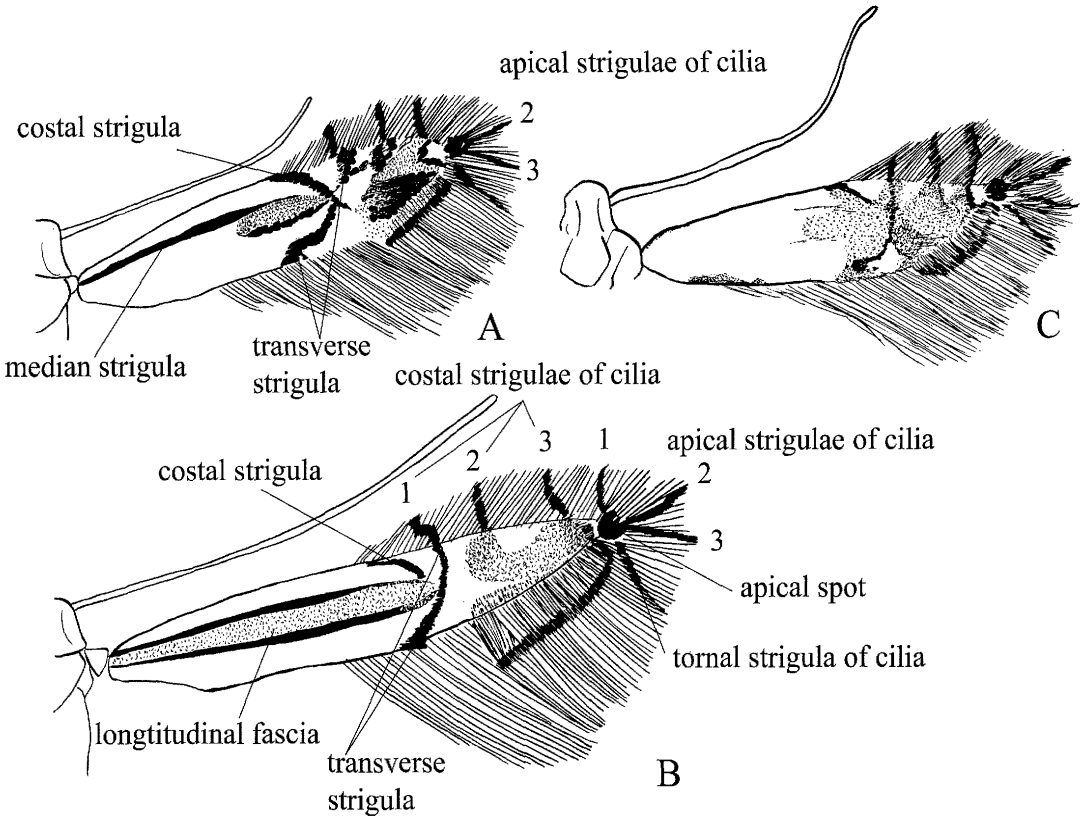

Fig. 3. Forewing fascia and strigulae of Saliceae mining *Phyllocnistis* species. A: *P. gracilistylella*. B: *P. saligna*. C: *P. unipunctella*.

species or allopatric populations of gracillariid species, although the construction of the library is still in progress. There are many studies attempted to identify or classify microlepidopteran groups using both morphological and DNA barcoding approaches (*e.g.* Emery *et al.*, 2009; Lees *et al.*, 2010). Some studies have revealed that some species complexes are not discriminated to the level of each species by DNA barcoding (Kaila and Ståhls, 2006; Langhoff *et al.*, 2009).

In Europe and Russia, some willow and poplar mining *Phyllocnistis* species are known (e.g. *Phyllocnistis saligna* (Zeller, 1839) and *P. unipunctella* (Stephens, 1834)) (Claridge and Wilson, 1982; Seksjaeva, 1989). Recently, *Phyllocnistis ramulicola*, which is very similar to *P. saligna* in wing pattern, has been recorded from the stems of *Salix* spp. from England and Portugal, (Langmaid and Corley, 2007).

In Japan, five *Phyllocnistis* species are known: (i) *P. selenopa* Meyrick, 1915; (ii) *P. citrella* Stainton, 1856; (iii) *P. saligna* (Zeller, 1839); (iv) *P. toparcha* Meyrick, 1918; and (v) *P. hyperbolacma* (Meyrick, 1931). In addition, some unnamed species have also been collected from several plants (Nakamura, 1993; Murase, 2005; Owada *et al.*, 2006; Arita *et al.*, 2009). Among them, *P. saligna* is known to feed on *Salix* spp. According to Hirano (pers. comm.), an unrecorded leaf-miner species occurs on *Salix* spp. in Nagano Prefecture and is distinguishable from *P. saligna* by pupal morphology.

In this paper, we describe the adult and pupal morphologies of a new species and of *P. saligna*, both of which are associated with *Salix* spp. in Japan. We also report their life histories. In addition, we compare molecular divergence among Japanese species, and assess relationships between Japanese and European populations, based on DNA barcode sequences.

Materials and Methods

Study sites. Specimens were collected from July to November in 2008 and from March to November in 2009 and 2010 in the following prefectures: Yamagata (Ohyama, Tsuruoka (38°44-45′N, 139°44-45′E, 10 m)), Nagano (Mt. Kasaga-dake, Takayama (36°40′22″N, 138°28′31″E, 1780 m; Fig. 1A); Saigawa Riv., Nagano (36°37′20″N, 138°11′39″E, 350 m; Fig. 1B); Azusa Riv., Matsumoto (36°13′00″N, 137°52′13″E, 630 m; Fig. 1C)), Aichi (Shidara (35°07′12″N, 137°28′54″E, 920 m)), Mie (Nabari Riv., Nabari (34°37′08″N, 136°06′08″E, 200 m; Fig. 1D)) and Nara (Shōren-ji Riv. from Mitsue to Soni (Soni: 34°30′33″N,

Table 1. Sampling information of *Phyllocnistis* species and GenBank accession numbers.

Species name	Host plant	Stem/leaf and upper/lower mines	Collection site	Voucher number	GenBank accession no. COI
Phyllocnistis gracilistylella	*S. gilgiana*	leaf/lower	Nagano, Nagano	SK-002	AB614504
P. gracilistylella	*S. integra*	leaf/lower	Matsumoto, Nagano	SK-004	AB614506
P. gracilistylella	*S. gilgiana*	leaf/lower	Nabari, Mie	SK-008	AB614508
P. gracilistylella	*S. gracilistyla*	leaf/lower	Soni, Nara	SK-010	AB614510
P. saligna	*Salix* sp.	stem	Nagano, Nagano	SK-001	AB614503
P. saligna	*S. integra*	stem	Matsumoto, Nagano	SK-003	AB614505
P. saligna	*S. gilgiana*	stem	Nabari, Mie	SK-007	AB614507
P. saligna	*Populus sieboldii*	leaf/upper	Nabari, Mie	SK-012	AB614512
P. saligna	*S. gracilistyla*	stem	Soni, Nara	SK-009	AB614509
P. saligna	*S. gracilistyla*	leaf/upper	Soni, Nara	SK-011	AB614511
P. citrella	*Citrus junos*	leaf/lower	Soni, Nara	SK-013	AB614513
P. citrella	*Citrus junos*	leaf/lower	Soni, Nara	SK-014	AB614514

136°07′23″E, 420 m; Fig. 1E–F); Nosegawa (34°09′N, 135°39′E, 1200 m)). Adult specimens preserved in the Entomological Laboratory, Osaka Prefecuture University (OPU) and those collected by N. Hirano (Matsumoto), were examined.

Leaf mine sampling and rearing. The larvae and cocoons were collected from leaves, branches, and trunks of host plants of ten Salix and one Populus species. They were reared in plastic cups (420ml: 129π × 60H) containing wet cotton at 20±5°C under a photoperiod of 13~16–h light: 8~12–h dark in the laboratory. For the leaf- and stem-miner species, the morphology of each instar and pupa was recorded. Samples of the larvae, pupae, and adults of some species were preserved in 99 % ethanol for DNA sequencing.

Photography and dissection. Photographs of leaf mines were taken in the field using an OLYMPUS μ1060 digital camera. Some pupae were dried and sputter-coated with a 60: 40 mixture of gold-palladium for examination with a scanning electron microscope (SEM). SEM photographs were taken using HITACHI SU1510 with a lanthanum hexaboride (LaB6) source at an accelerating voltage of 15 kV. For preparation of the male and female genitalia, the abdomen was removed and boiled for 3-4 min in 10% aqueous KOH. They were stained with acetocarmine.

Specimen deposition, nomenclature, and diagnosis. All the examined specimens are deposited in OPU. Scientific names of plants follow Missouri Botanical Garden (2010). Because adults do not present any obvious external sexual dimorphism as pointed out in *Phyllocnistis citrella* (Jacas and Garrido, 1996), we treat specimens without dissection as sex unknown. Adult wing pattern nomenclature follows Kawahara *et al.*, (2009: fig. 3), and the genital structure nomenclature follows Kawahara *et al.* (2009) and Kuroko (1982).

Molecular analysis

We tried to compare molecular divergence among Japanese species, but we could not obtain fresh materials for *Phyllocnistis unipunctella*. We focused in this study on two Salicaceae mining species which occurred together (a new leaf miner, *Phyllocnistis gracilistylella* sp. nov., and a stem miner, *P. saligna*), in order to compare their molecular divergence, and we sampled both species from four localities; Nagano, Matsumoto, Mie, and Nara. *Phyllocnistis citrella* was selected as an outgroup (Table 1). Total DNA was extracted from middle and hind legs of adult specimens by using DNeasy Tissue Kit (Qiagen) following the User's Guide. Used specimens were stored in 99 % ethanol or dried.

For molecular phylogenetic inference, the DNA barcode region, a part of the mitochondrial COI gene was chosen. This gene is known to be useful for inferring relationships among closely related moth species and populations (Brown *et al.*, 1994). Primer sets LCO1490 (fwd) (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (rev) (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.*, 1994) were used to amplify a 658 bp fragment of mitochondrial COI.

The aligned sequences were subjected to a neighbour-joining (NJ) analysis using PAUP Ver. 4.0 b 0 (Swofford, 2002) to provide genetic relationships among species or populations. the bootstrap values were estimated using 1000 replicates with Kimura's two-parameter model of substitution (K2P distance) evolution model. Sequence data in this study were also compared with other Salicaceae mining *Phyllocnistis* species registered in the official DNA barcode library, using the BOLD identification system (IDS), an identification interface using the DNA barcode library available from the BOLD website (http://www.barcodinglife.org/) [accessed 1 October 2010].

Results and discussion

Species descriptions

Diagnostic features of the Japanese Salicaceae mining *Phyllocnisits* species are summarized in Table 2.

Phyllocnistis gracilistylella Kobayashi, Jinbo & Hirowatari **sp. nov.**
(Japanese name "Neko-yanagi-Kohamoguri")
(Figs 2A-B, 3A, 4A-D, 6, 8A-D, 9, 10)

Diagnosis. Forewing lustrous-white with one fuscous median strigula and another thin fuscous median strigula, enclosing a short orange-ocherous fascia; apical area mixed with blackish fuscous (Table 2, Figs 2A-B, 3A). Male genitalia with a nearly spoon-shaped valva; valva slightly broadened 1/4 to apex, rounded apically (Figs 4A-B). Corpus bursae with a pair of similarly sized signa, each with a long spine protruding at its center (Fig. 4D).

Description. Wing expanse 5.8 mm in holotype, 4.5–6.0 mm in paratypes. Frons lustrous white. Antennae lustrous white, slightly mixed with golden scales. Thorax lustrous white. Abdomen silver-gray or ocherous-gray. Anal tuft white.

Forewing (Figs 2A-B, 3A). Lustrous-white to silver-white, apical 1/3 variably suffused with pale gold to Indian yellow, with blackish fuscous patches; one fuscous median strigula from base to 2/3, another thin fuscous median strigula from 1/2 to 2/3, the two enclosing an orange-ocherous fascia; a fuscous costal strigula from 3/5 to middle, an obscure oblique fuscous transverse strigula from costal 4/5 to dorsal 1/2. Cilia white with three fuscous costal strigulae before apex; a black apical spot, giving origin to four divergent fuscous apical strigulae, one extending to upper part of costal cilia, but sometimes indistinct, the second and third to apex, the fourth (tornal) to upper part of terminal cilia; terminal cilia white with a fuscous fringe line near termen. Hindwing pale gray to white; cilia white.

Male genitalia (Figs 4A-C). Uncus absent. Tuba analis broad, weakly sclerotized. Valva long and slender, nearly spoon-shaped, broaded 1/4 to apex, slightly broad toward dorsal side. Vinculum U-shaped. Aedeagus simple, elongate, tapering caudally. Transtilla present. Coremata present on both sides of Segment 8.

Female genitalia (Fig. 4D). Apophyses anteriores and apophyses posterores slender. Ostium bursae and ductus bursae slender, membranous; inception of ductus seminalis on the anterior side of corpus bursae. Corpus bursae elongate, membranous; signa a pair of similar sized sclerites, each with a long spine protruding in the center.

Pupa (Figs 6J-L, 8A-H). Dark brown, 3–4 mm in length, ~0.8 mm in diameter. Vertex with a long, stout, acute frontal process (cocoon cutter) (Figs 8A-D). Dorsum of A2-A7 with a pair of slightly laterally curved, large spines in between which several small spines projecting centrally (Figs 8E-G). A10 with a pair of slightly bifurcate processes from caudal apex (Fig. 8H).

Distribution. Japan: Honshu (Yamagata, Nagano, Nara, and Mie Prefectures), Kyushu (Fukuoka Pref.).

Host plant. *Salix gracilistyla* Miq., *S. serissaefolia* Kimura, *S. integra* Thunb., *S. gilgiana* Seemen. (Salicaceae).

Material examined—116 (7♂5♀104exs)

Type Material. Holotype ♂, JAPAN: Honshu, Shōrenji-River, Imai, Soni, Uda, Nara Pref., 23. x. 2008 em. (S. Kobayashi), Host: *Salix gracilistyla*, 13. x. 2008 (larva), (genitalia slide no. OPU-SK131). Paratypes 6♂5♀104 exs. [Host: *S. gracilistyla*]: [Same locality as holotype, S. Kobayashi leg.]: (leaf mine of lower epidermal cell): Nara Pref.: 2♂2♀3exs, 16-27. x. 2008 em., 13. x. 2008 (larva); 16exs, 15-18, 20. x. 2009 em., 4. x. 2009 (larva) (AB614510); 7exs, 14, 21. x. 2010 em., 2. x. 2010 (larva). 1ex, Yokowa, Imai, Soni, Uda, 5. viii. 2009 em., 12. vii. 2009 (larva); 1♀2exs, Same locality, 6, 13, 18. x. 2009 em., 27. ix. 2009 (larva).; (leaf mine of upper epidermal cell): 1ex, 18. x. 2008 em., 13. x. 2008 (larva).

[Host: *S. serissaefolia*]: Yamagata Pref.: 1exs, Oyama, Tsuruoka, 21. ix. 2010 em. (S. Kobayashi), 18. ix. 2010 (larva). Nagano Pref.: 3♂38exs, Azusa-River, Nagawa, Matsumoto, 1-15. x. 2008 em. (S. Kobayashi, T. Hirowatari & K. Ikeuchi), 1. x. 2008 (larva).

[Host: *S. integra*]: 1♂2♀11exs, Same locality, 2-15. x. 2008 em. (S. Kobayashi, T. Hirowatari & K. Ikeuchi), 1. x. 2008 (larva) (AB614506); [H. Kuroko leg.]: 12exs, Sagadaira, 11&16. ix. 1963. 3exs, Tateshina-Kogen, 19. ix. 1965.

[Host: *S. gilgiana*]: [S. Kobayashi leg.]: (leaf mine of lower epidermal cell): Nagano Pref.: 8exs, Saigawa, Nagano, 14-16, 19, 20-23, 26-28. ix. 2009 em., 11. ix. 2009 (larva) (AB614504); Mie Pref.: 1ex, Natsumi, Nabari, 16. x. 2009 em., 3. x. 2009 (larva) (AB614508).

Etymology. The specific epithet, *gracilistylella*, derives from the host plant, *Salix gracilistyla*.

Biology (Figs 6, 9Aa-b, Ba-b, Ca-b). This species has a few generations per year. The larvae emerged in July and October to November in Nara Prefecture. The exact number of larval instars was not detectable. The larva, presumably from first to fourth instars, is a sap-feeding leaf miner, forming an elongate tortuous, upper or lower surface, serpentine mine (~50–150 mm in length; ~1–2 mm in

Table 2. Diagnostic features of three Salicaceae mining *Phyllocnistis* species in Japan.

Species name	Host plant	Adult								Pupa
		Costal strigula	Longitudinal fascia	Transverse strigula	Costal strigulae of cilia	Apical strigulae of cilia	Apical spot	Valva	Signa	Cocoon cutter
P. gracilistylella	*Salix gracilistyla, S. integra, S. gilgiana, S. serissaefolia*	Broad	Apical 1/3 of median strigula	Broad, oblique	3	3, 1st strigula sometimes absent	1	~1.8 × length of vinculum, long and slender, nearly spoon-shaped	Paired, similar in shape	Developed, slender
P. saligna	*Salix* spp., *Populus sieboldii*	Slender	Broad, yellow	Slightly curved	3	3	1	~2.0 × length of vinculum, slender and tapering to apex, apically curved ventrally	Two, dissimilar in shape	Reduced, curved, flat
P. unipunctella	*Populus nigra* var. *italica*	Very slender, black line	Indistinct, yellow	J-shaped, indistinct	3	3, 1st indistinct	1	~1.9 × length of vinculum, slender and broaded from 1/4 to apex and curved ventrally	Paired, similar in shape	Not examined

width); 1–2 mines were usually observed in a leaf.

Host ranges and mine positions of the new species were restricted to leaves of four *Salix* species (i.e. *S. gracilistyla*, *S. serissaefolia*, *S. gilgiana* and *S. integra*), while all the stem-mining larvae were identified as *P. saligna* (Table 3).

In *Salix integra*, *S. gilgiana* and *S. serissaefolia*, only leaf mines were observed on the lower side epidermis cells (Figs 6N-O, Q, 9Ba-b, Ca-b). Among them, a dark median frass line was obvious in the mines in *S. integra* (Figs. 6N-O). Exceptionally in *S. gracilistyla*, larval mines were observed on both sides of the leaf (Table 3, Fig. 9Aa-b). Mines of the upper epidermis cell are clear grayish, very conspicuous, like fly larva mines (Figs 6A, C, E). The final instar larva spun a white cocoon at the leaf margin or base of the leaf, the leaf margin slightly curled upwards by contraction of the cocoon silk (Figs 6I, O, Q). Many mines of the new species were found on young leaves or from young branches close to the ground up to ~1 m along water edges.

In *S. gracilistyla*, upper surface leaf mines were observed as many as lower surface mines, sometimes more than lower surface ones. Most upper surface mines were formed by young larvae of *P. saligna* and rarely by the new species (Figs 6A, C, 7A-B).

Remarks. The genital structure of this species is similar to that of some leaf miners of poplar, aspen and willows (e.g. *P. valentinensis* Hering, 1936 and *P. unipunctella* (Stephens, 1834)), which may indicate a close relationship to them. But this new species is distingushed by the U-shaped vinculum in the male genitalia (Fig. 4B) and the median strigula, enclosing an orange-ocherous fascia in 1/2 of the forewing (Table 2, Fig. 3A).

Phyllocnistis saligna (Zeller, 1839)
(Japanese name "Yanagi-Kohamoguri")
(Figs. 2C-D, 3B, 4E-H, 7, 8I-O, 9, 10)

Opostega saligna Zeller, 1839, *Isis*. 32: 214; *Phyllocnistis saligna* (Zeller, 1839): Kuroko, 1982, 202, Pl. 5–2, 273–8; Ermolaev, 1987, 37; Seksjaeva, 1989, 402; Seksjaeva, 1997, 429–430; Parenti, 2000: Pl. 47; Langmaid and Corley, 2007, 234–235, figs. 2, 7, 10.

Tinea cerasifoliella Hübner, 1796, *Samml. eur. Schmett.*: 1–78, pls. 1–71. (Nomen oblitum)

Phyllocnistis salignatella Bruand, 1851, *Mém. Soc. Emul. Doubs* 3 (1849) (5–6): 23–68; Phyllocnistis salignella Herrich-Schäffer, 1855, *Syst. Bearbeitung Schmett. Eur.*: 1–394. (Unjustified emendation)

Phyllocnistis lugdunensella Bruand, 1858, *An. Soc. Entomol. Fr.* (3 Série) 6: 691.

Phyllocnistis asiatica Martynova, 1955, *Entomol. Obozr.* 34: 248, fig. 1b.

Diagnosis. Forewing elongate, lustrous-white with a pair of fuscous median strigulae, enclosing an orange-ocherous longitudinal fascia about half of wing length; apical area yellowish gold (Table 2, Figs 2C-D, 3B). Male genitalia with slender valva, tapering to apex, apically curved ventrally, with slender basal processes (Figs 4E-F). Corpus bursae with two signa; posterior with a bifurcate spine and small spines, anterior with a long spine and small spines (Fig. 4H).

Description. Wing expanse 6.0–7.0 mm.

Forewing (Figs. 2C-D, 3B). Male genitalia (Fig. 4E-G). See Seksjaeva (1989) and Kuroko (1982). Female genitalia (Fig. 4H) Pupa (Figs. 7J-L, 8I-O). Ocherous to brown, 4 mm in length, ~1 mm in diameter. Vertex with a slightly dorsally curved, spine-like process (cocoon cutter) (Figs 8I-L). Dorsum of A2-A7 with a pair of claw-shaped large spines in between which several small spines projecting centrally (Figs 8M-N). A10 with a pair of slightly bifurcate processes from caudal apex (Fig. 8O).

Distribution. Japan: Hokkaido, Honshu, Shikoku, Kyushu; China, India (Kuroko, 1982), Central Asia, Russia, Europe (Seksjaeva, 1989).

Host plant. *Salix gracilistyla* Miq., *S. integra* Thunb., *S. sachalinensis* Fr. Schmidt., *S. gilgiana* Seemen., *S. bakko* Kimura, *S. reinii* Franch. & L. Sav., *S. sieboldiasna* Seemen, *S. serissaefolia* Kimura, *S. subfragilis* Andersson, *S. babylonica* L., *S. chaenomeloides* Kimura, *Populus sieboldii* Miq. (Salicaceae).

Table 3. Mining positions of *Phyllocnistis gracilistylella* and *P. saligna* on ten Salicaceae species.

Host plants	Species name	
	Phyllocnistis gracilistylella	*P. saligna*
Salix gracilistyla	+ L (u/l)	+ S, L (u)
S. integra	+ L (l)	+ S
*S. sachalinensis**	-	+ S
S. gilgiana	+ L (l)	+ S
S. bakko	-	+ S
S. reinii	-	+ S
S. serissaefolia	+ L (l)	+ S
S. subfragilis	-	+ S
S. chaenomeloides	-	+ S
S. babylonica	-	+ S
Populus sieboldii	-	+ S, L (u)

+: larvae were examined; -: larvae was not examined. The alphabet before parenthesis refers to plant tissue feeding by larva as follows: L: leaf epidermis; S: stem epidermis. The alphabet shown in the parenthesis refer to the leaf feeding point as follows; l: lower surface; u: upper surface. *Leaf mines of upper surface were collected, but no adult moth was emerged.

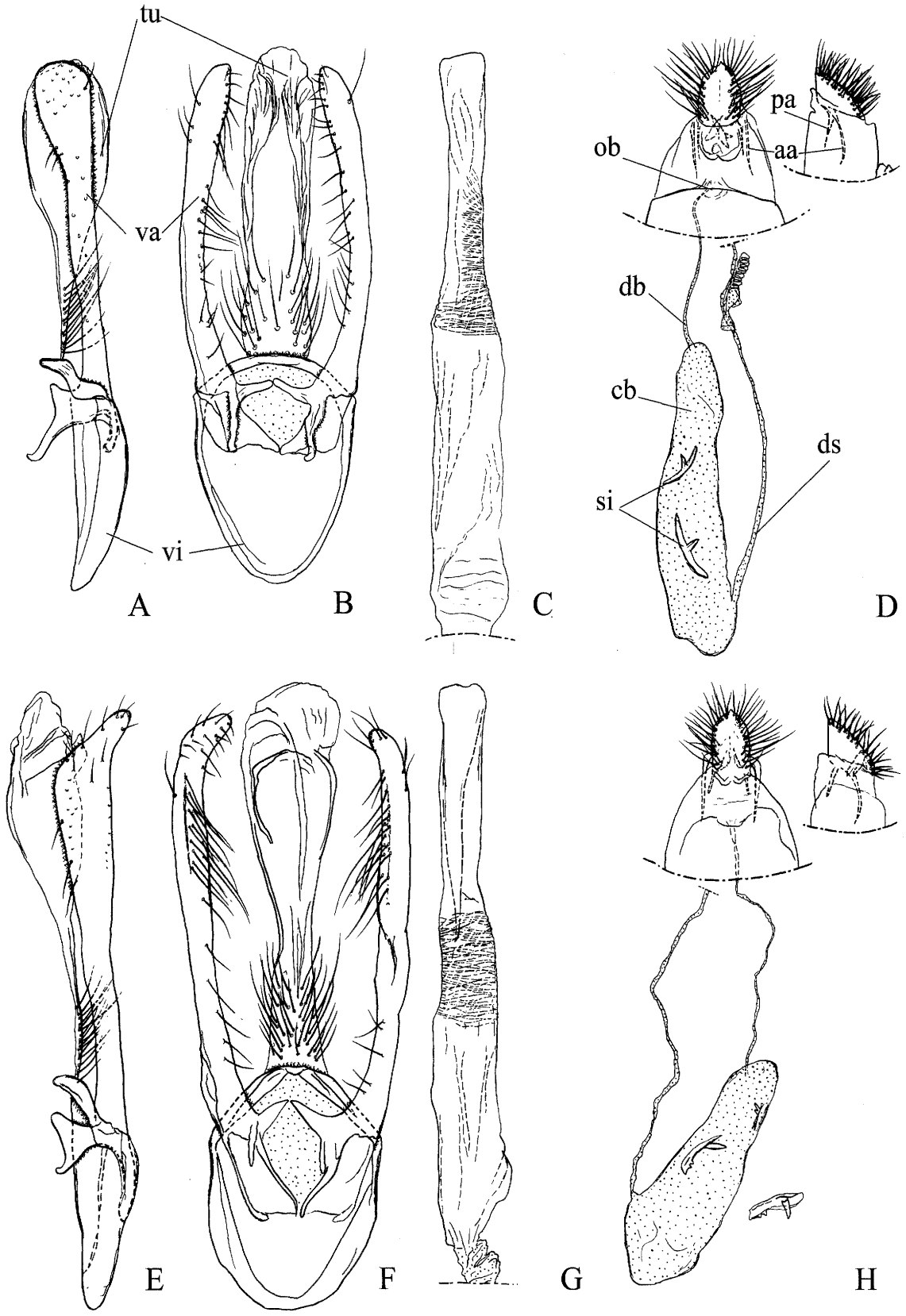

Fig. 4. Genitalia of *Phyllocnistis gracilistylella* and *P. saligna*.
A–D: *P. gracilistylella*; E–H: *P. saligna*. A, E: Male genitalia, latetral view. B, F: *Ditto*, ventral view. C, G: Aedeagus, ventral view. tu: tuba analis; va: valva; vi: vinculum. D, H: Female genitalia, ventral view. aa: anterior apohyses; pa: posterior apophyses; ob: ostium bursae; db: ductus bursae; cb: corpus bursae; si: signa; ds: ductus seminalis.

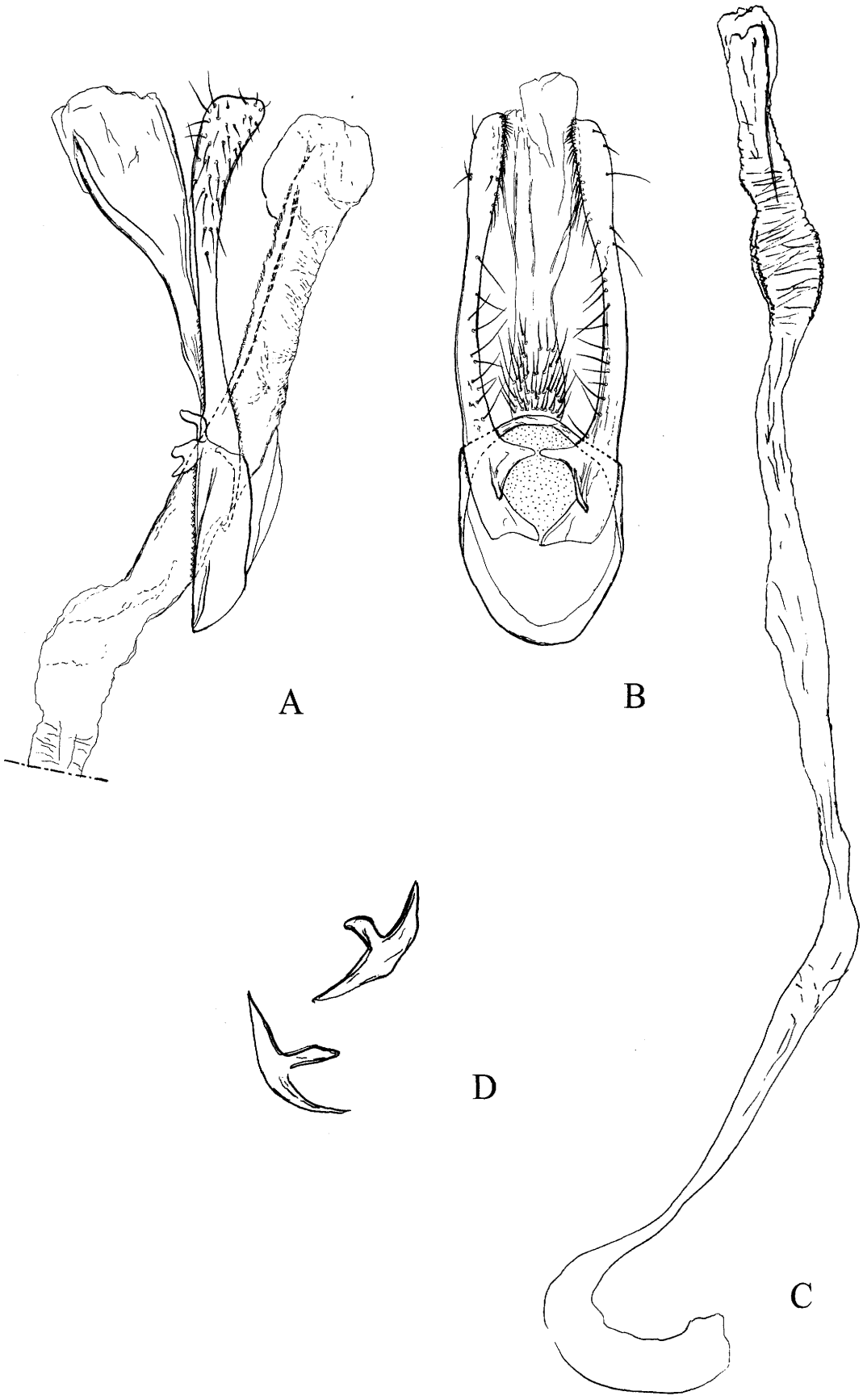

Fig. 5. Genitalia of *Phyllocnistis unipunctella*.
A: Male genitalia, latetral view. B: *Ditto*, ventral view. C: Aedeagus, ventral view. D: Signa of female genitalia.

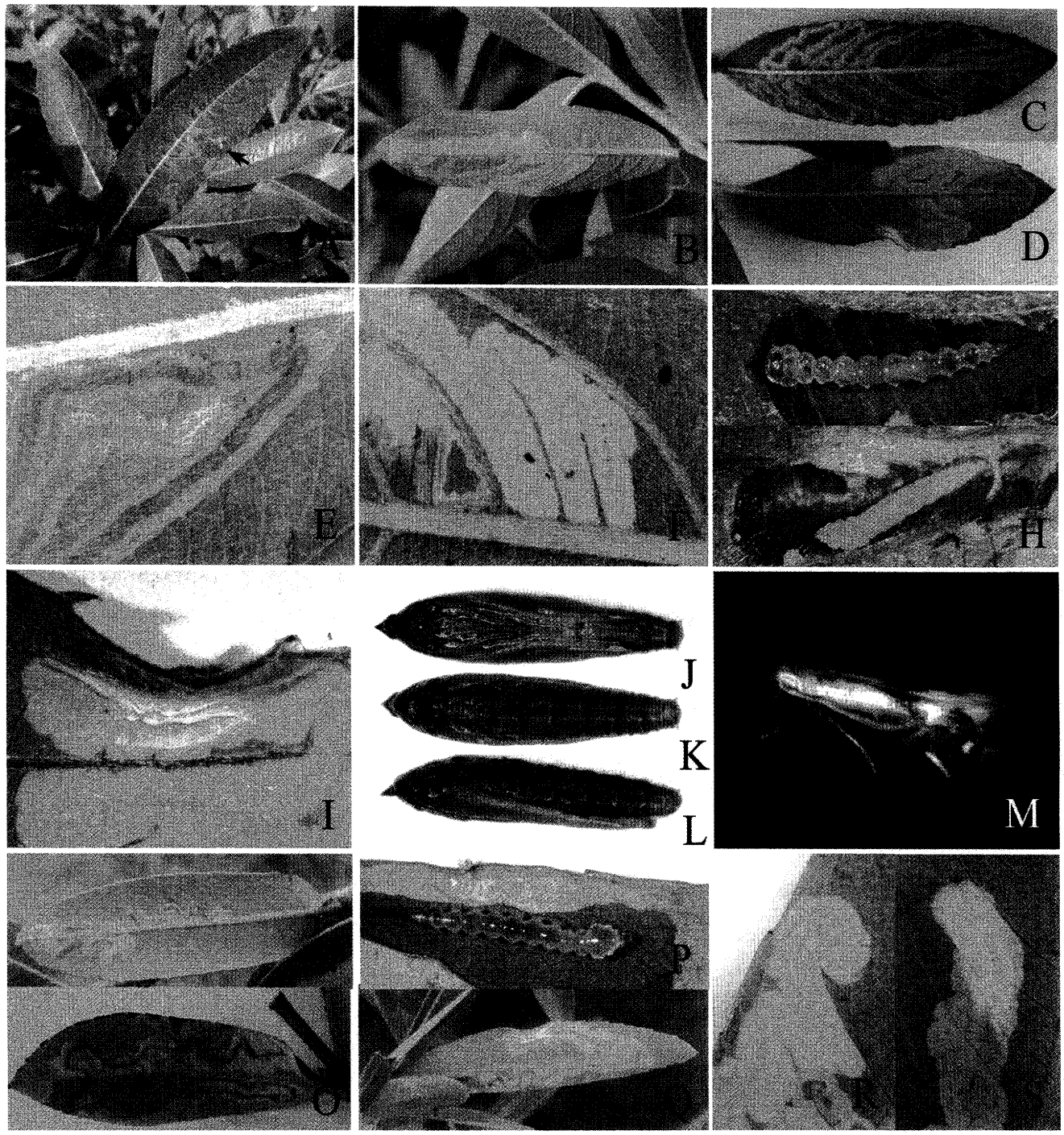

Fig. 6. Immature stages of *Phyllocnistis gracilistylella* and the host plants.
A–M: Host: *Salix gracilistyla*. N–P: *S. integra*. Q–S: *S. gilgiana*. A, C. Mine of upper surface of leaf by later larva. B, D. Mine of lower surface of leaf by later larva. E. Later instar larva on upper surface of leaf. F, N, R. Later instar larva on lower surface of leaf. G, P. Mature sap-feeding larva. H. Final instar spinning larva. I, O, Q, S. Pupal chamber and leaf mine on lower surface of leaf. J. Pupa (ventral view). K. *Ditto* (dorsal view). L. *Ditto* (lateral view). M. Resting posture of the adult.

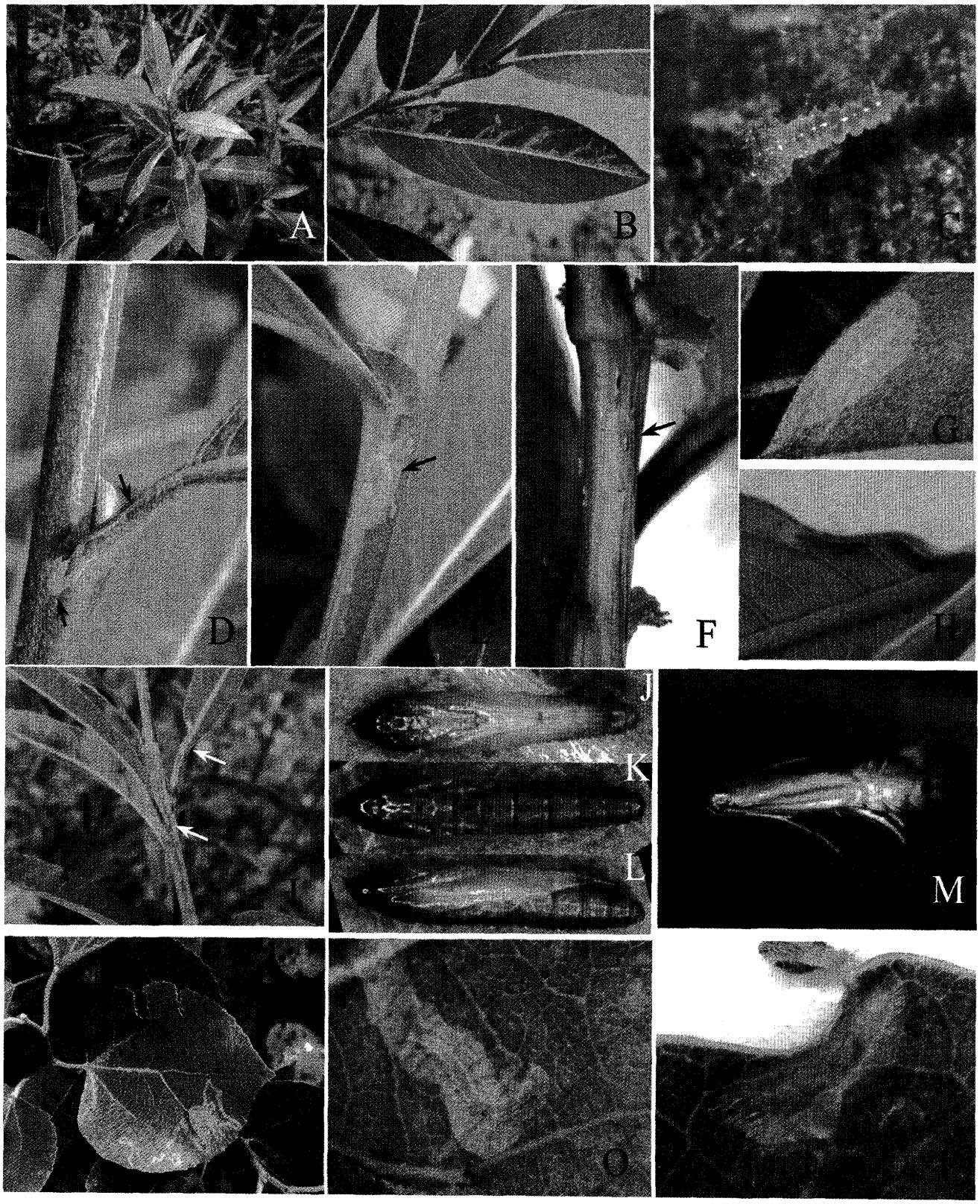

Fig. 7. Immature stages of *Phyllocnistis saligna* and the host plants.
Host: A–E, G, H, J–L: *Salix gracilistyla*. F: *S. chaenomeloides*. I: *S. gilgiana*. M: *S. sachalinensis*. N–P: *Populus sieboldii*. A. Host plants and leaf mines formed by young larvae. B. Young mine of upper surface of leaf. C. First instar larva. D. Stem mine via leafstalk. E, F. Stem mine and later larva. G. Final instar larva mines upper leaf blade. H, P. Pupal chamber. I. Stem mine and pupal chamber. J. Pupa (ventral view). K. *Ditto* (dorsal view). L. *Ditto* (lateral view). M. Resting posture of the adult. N. Leaf mine by young larva. O. Mature sap-feeding larva.

Material examined—130 (2♂3♀125exs)

[Host: *S. gracilistyla*]: Nagano Pref.: 1ex, Oshirakawa, Nagawa, Matsumoto, 7. x. 2008 em. (S. Kobayashi & T. Hirowatari), 1. x. 2008 (larva). Aichi Pref.: 8exs, Kirara, Shidara, 6, 15. x. 2008 em. (S. Kobayashi & T. Hirowatari), 30. ix. 2008 (larva). Shiga Pref.: 5exs, Umegahara, Maibara, 8, 13, 16, 18-26. x. 2009 em. (S. Teramura), 4. x. 2009 (larva). Nara Pref. [S. Kobayashi leg.]: [Shorenji-River, Imai, Soni, Uda]: 2♂1♀2exs, 16-17&23. x. 2008 em., 12. x. 2008 (larva); 5exs, 19-20. x. 2008 em., 13. 2008 (larva); 1♀6exs, 16-23. x. 2008 em., 13. x. 2008 (larva); 1ex, 4. vii. 2009 em., 28. vi. 2009 (larva); 3exs, 30. ix. 2009 em., 27. ix. 2009 (larva); 8exs, 1, 5-6. x. 2009 em., 27. ix. 2009 (larva); 3exs, 7-8. x. 2009 em., 4. x. 2009 (larva) (AB614509); (leaf mine of upper epidermal cell): 1ex, 15. ix. 2009 em., 30. viii. 2009 (larva) (AB614511). [Yokowa, Soni, Uda]: 1ex, 30. vii. 2008 em., 27. vii. 2008 (larva). 1ex, 29. vii. 2009 em., 26. vii. 2009 (pupa); 2exs, 30-31. vii. 2009 em., 18. vii. 2009 (larva). [Kuzure-dani, Momonomata, Mitsue]: 4exs, 28, 30. vii., 1-2, 4. viii. 2009 em., 26. vii. 2009 (larva). 1ex, Tateriko, Nosegawa, 3. viii. 2008 em. (S. Kobayashi & T. Hirowatari), 29. vii. 2008 (larva). Fukuoka Pref.: [Hikosan, H. Kuroko leg.]: 3exs, 4. xi. 1954; 4exs, 15-19. vi. 1955; 1ex, 24. vi. 1955.

[Host: *S. integra*]: Nagano Pref. [S. Kobayashi, T. Hirowatari & K. Ikeuchi leg.]: 2exs, Azusa-River, Nagawa, Matsumoto, 4. x. 2008 em., 1. x. 2008 (larva).

[Host: *S. sachalinensis*]: [S. Kobayashi leg.]: Yamagata Pref.: [Shimo-ike, Oyama, Tsuruoka, 18. ix. 2010 (larva, pupa)]: 2exs, 21, 27. ix. 2010 em.; (leaf mine of upper epidermal cell): 3exs, 23. ix., 1. x. 2010 em.; Mie Pref.: 1ex, Dodo, Kamiya, Nabari, 27. vii. 2009 em., 25. vii. 2009 (pupa). Nara Pref.: 1ex, Kuzure-dani, Momonomata, Mitsue, 28. vii. 2009 em., 18. vii. 2009 (larva); 2exs, Same locality, 1-2. vii. 2009 em., 26. vii. 2009 (larva).

[Host: *S. serissaefolia*]: Yamagata Pref.: 1exs, Oyama, Tsuruoka, 22. ix. 2010 em. (S. Kobayashi), 18. ix. 2010 (larva). Nagano Pref. [S. Kobayashi, T. Hirowatari & K. Ikeuchi leg.]: 1♀5 exs, Azusa-River, Nagawa, Matsumoto, 3-10. x. 2008 em., 1. x. 2008 (larva).

[Host: *S. subfragilis*]: [S. Kobayashi leg.]: 2exs, Shorenji-dum, Shorenji, Nabari, Mie, 1-2. vii. 2009 em., 27. vi. 2009 (larva); 2exs, Same locality, 13, 16. vii. 2009 em., 11. vii. 2009 (larva). 1ex, Shioi, Soni, Uda, Nara, 5. x. 2009 em., 26. ix. 2009 (larva).

[Host: *S. bakko*]: Nagano Pref.: [S. Kobayashi leg.]: 6exs, Saigawa, Nagano, 14-20. ix. 2009 em., 11. ix. 2009 (larva). 3exs, Yamada pasture, Takayama, 16, 20. ix. 2009 em. (S. Kobayashi & H. Tsuruta), 11. ix. 2009 (larva).

[Host: *S. reinii*]: 2exs, Kasagadake, Yamada, Nagano, 5-6. viii. 2010 em. (S. Kobayashi), 2. viii. 2010 (larva).

[Host: *S. sieboldiasna*]: Fukuoka Pref.: [Hikosan, H. Kuroko leg.]: 4exs, 4, 8. viii. 1960; 3exs, 27. x. 1960; 4exs, 2, 9. xi. 1960.

[Host: *Salix* sp.]: [S. Kobayashi leg.]: Yamagata Pref.: 3exs, Shimo-ike, Oyama, Tsuruoka, 22-24. ix. 2010 em., 18. ix. 2010 (larva). Nagano Pref.: 15exs, Saigawa, Nagano, 16-24. ix. 2009 em., 11. ix. 2009 (larva) (AB614503). Tokushima Pref. 2exs, Tsurugi-san, Naga, 29. viii. 2010 em., 22. viii. 2010 (larva).

[Host: *Populus sieboldii*]: [S. Kobayashi leg.]: Mie Pref.: [Kajikabashi, Kochidani, Nabari]: 1ex, 20-22. vii. 2009 em., 11. vii. 2009 (larva); 2exs, 27. vii. 2009 em., 19. vii. 2009 (larva) (AB614512); 1ex, 6-7. ix. 2009 em., 30. viii. 2009 (larva). 1ex, Kameyama, Taroji, Soni, Uda, Nara, 4. ix. 2009 em., 31. viii. 2009 (larva).

Yamagata Pref.: 1exs, Oyama, Tsuruoka, 18. ix. 2010 (S. Kobayashi), Adult on *S. serissaefolia*.

Biology (Figs 7, 9Ac-d, Bc-d, Cc-d). Kuroko (1982) recorded that in Japan the larva of this species mines from the stem, via petiole, to the leaf where it pupates on the basal leaf edge. But studies on the life history of *P. saligna* in Europe have noted that the larva first mines a leaf, then via the petiole, mines the twig, usually upward, subsequently entering another leaf via the petiole, where mining continues and a cocoon is made at the margin of the leaf (Hering, 1951; Langmaid and Corley, 2007). Seksjaeva (1989) recorded that larvae also mine leaves of poplars and willows. In the present study, we confirmed that the larva of this species in Japan firstly mines the leaf and later the stem and then moves, via a petiole, to a leaf, where it pupates on the basal leaf edge (Fig. 9Ac-d).

In *Salix gracilistyla*, *S. gilgiana* and *S. sachalinensis* the mine began from the middle of a lateral rib on the upper side of the leaf, and continued, sometimes tortuously, via the mid rib towards the base of the leaf (Figs 7B, D). In *S. subfragilis*, the mine began from a lateral leafstalk or the base of a mid rib or rarely near the mid rib on the lower surface of the leaf. The young larva is pale yellow, about 1.0 mm in length, and mines on the mid rib towards the lateral side of the leafstalk. The mine into the stem curves mostly upwards, and finally up the petiole of a leaf, ending at the leaf-base where the cocoon is spun (Figs 9Ac-d, Bc-d, Cc-d). Leaf mining larvae of this species were exceptionally observed in *S. gracilistyla* and *Populus sieboldii* (Figs 7A, N-O). In both cases leaf mines were only observed in the upper epidermis cell. In *P. sieboldii*, leaf mines are clear whitish, like fly larva mines; later the larva is yellow-green, 6-6.5 mm in length, and the cocoon

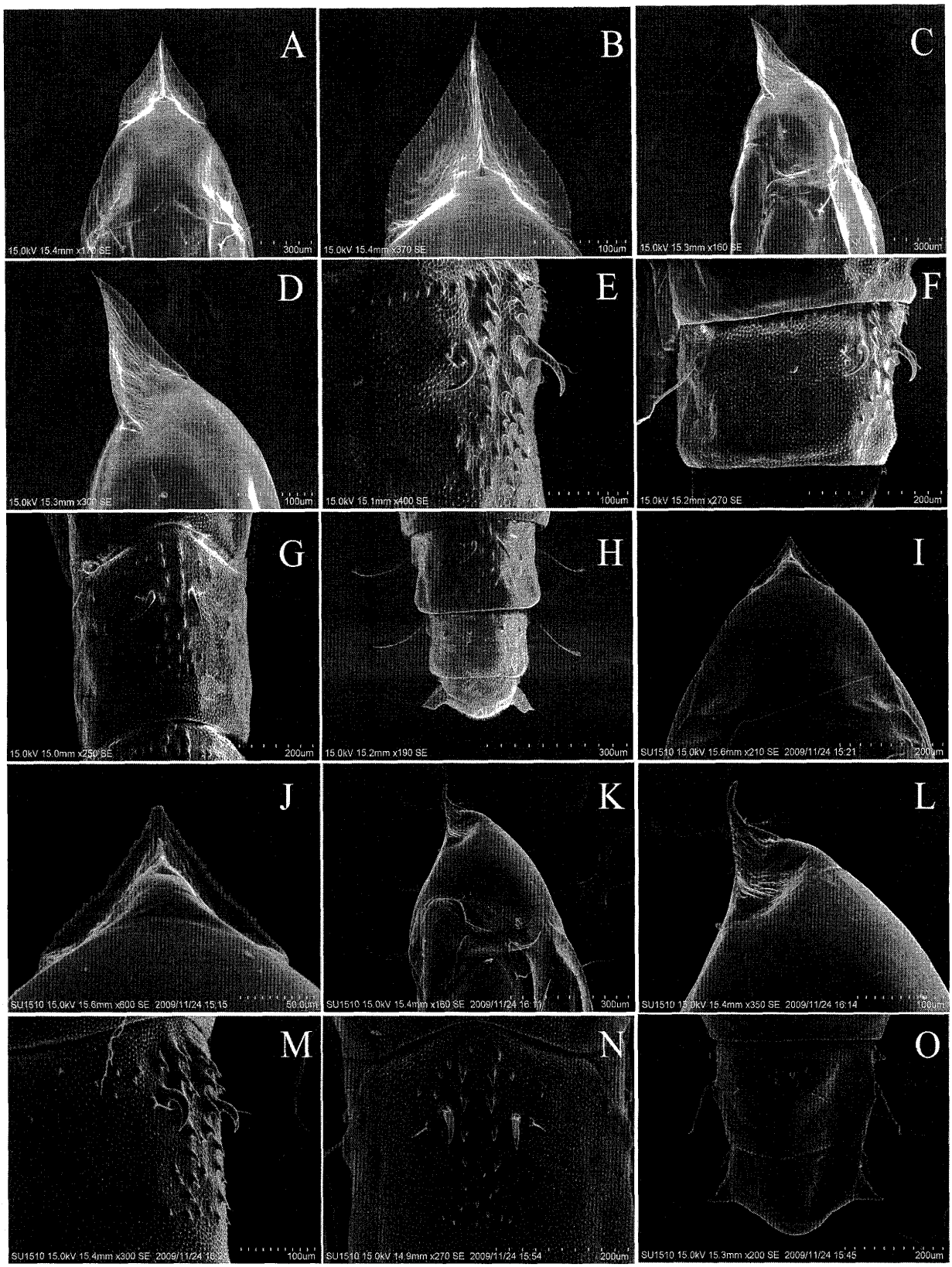

Fig. 8. Pupae of *Phyllocnistis gracilistylella* and *P. saligna*.
A–H: *P. gracilistylella*. I–O: *P. saligna*. A, I: Dorsal view of head. B, J: Ventral view of cocoon cutter. C, K: Lateral view of head. D, L: Lateral view of cocoon cutter. E: Spines of sixth abdominal tergum. F: Lateral view of eighth abdominal tergum. G: Dorsal view of fifth abdominal tergum. H: Dorsal view of A8-10. M: Lateral view of fifth abdominal tergum. N: Spines of third abdominal tergum. O: Dorsal view of A9–10.

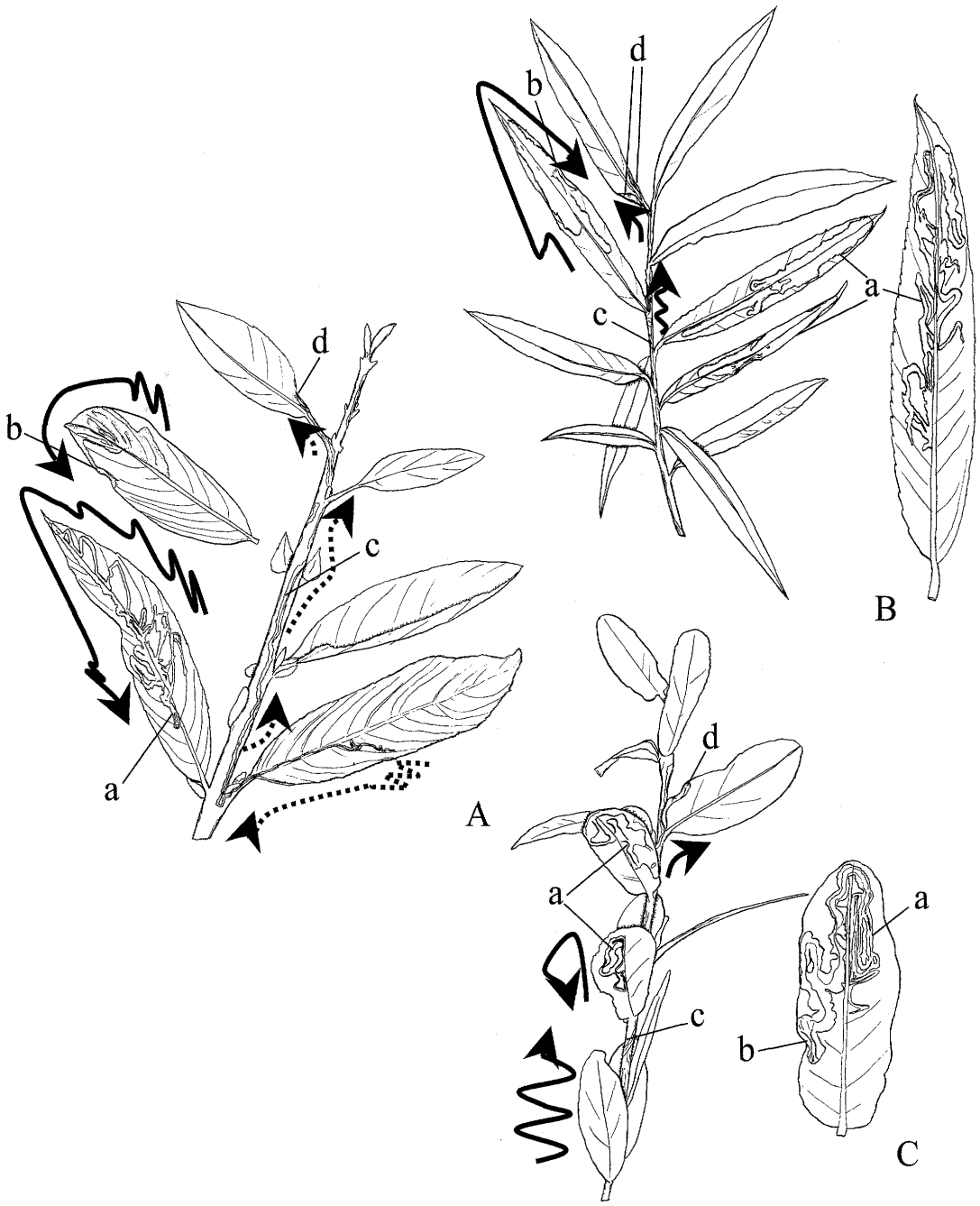

Fig. 9. Host plants and immature stage biology of *Phyllocnistis gracilistylella* and *P. saligna*.
A: On the leaf of *Salix gracilistyla*. B: On the leaf of *S. serissaefolia* C: On the leaf of *S. integra*. a–b. *P. gracilistylella*, c–d. *P. saligna*. (a) The larva forming serpentine mine on the epidermis of leaf. (b) Pupal cocoon fold on the leaf. (c) The larva forming long linear stem mine. (d) Pupal cocoon fold on the base of leaf. Arrows showing larval transfer.

is ocherous-white, 3-4 mm in length, 1 mm in width.

In the present study, we observed that the new species, *P. gracilistylella*, and *P. saligna* occurred together on the host plants. In many study sites, a complex pattern of stem mines was formed by many larvae of *P. saligna*, and a complex of leaf mines was formed on both sides of the leaf by larvae of the new species and of *P. saligna*.

Remarks. The forewing pattern and genital structure of Japanese specimens of *P. saligna* (Table 2) are very similar to those of *P. ramulicola*, in the following points: 1) the longitudinal fascia (basal fascia) often reaches the first costal strigula (Fig. 3B); 2) the valva of the male genitalia is not spoon-shaped, but curved inwardly at apex and tapering (Figs 4E–F); 3) the two signa of the female

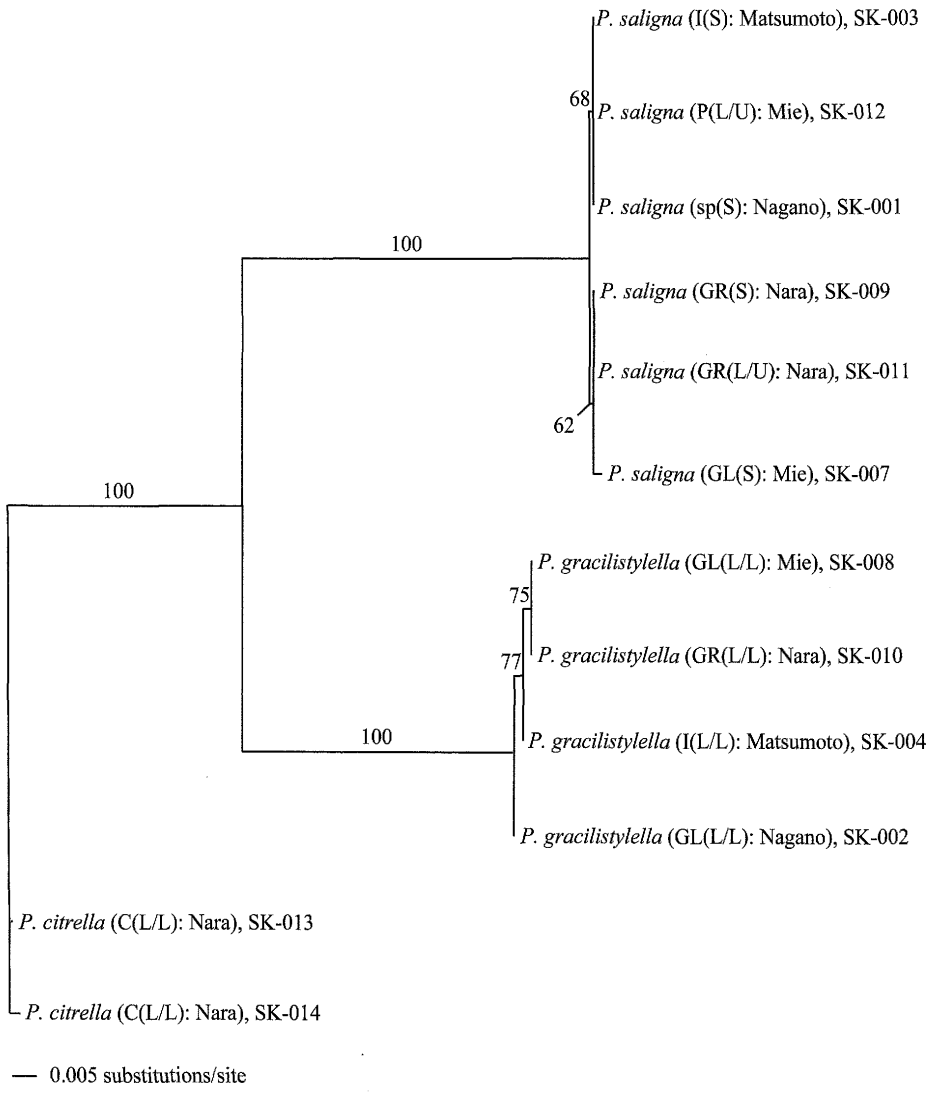

Fig. 10. A tree estimated by neighbor-joining analysis using the partial sequence of COI (658 bp) under the Kimura two-parameter distance model. Numbers above branches indicate bootstrap values (1000 replications). Branch lengths are proportional to distances. Within sample names, the first letters within the brackets refer to the scientific name of each host plant as follows: I: *Salix integra*; P: *Populus sieboldii*; sp: *Salix* sp.; GR: *S. gracilistyla*; GL: *S. gilgiana*; C: *Citrus junos*. The letters within the internal set of brackets preceding the forward slash refer to the mining position; L: leaf; S: stem. The letters after the slash refer to the mining side of the leaf; U. upper surface of leaf; L: lower surface of leaf.

genitalia are not equal in size (Fig. 4H). On the other hand, European specimens of *P. saligna* have characters which differ in the following points: 1) the longitudinal fascia does not reach the costal strigula; 2) the valva is spoon-shaped; 3) the two signa are equal in size (Langmaid and Corley, 2007). But these characters are not sufficient to identify the Japanese specimens as a separate species. The life history of *P. ramulicola* differs from that of *P. saligna* in that the larva of the former firstly mines into the stem. *Phyllocnistis canariensis* is also known to have the same mining habit as the larva of *P. saligna* (Klimesch, 1978), but it is distinguishable from *P. saligna* by having the longitudinal fascia in the forewing narrowly interrupted (Langmaid and Corley, 2007).

The results of molecular analysis indicate that Japanese representatives of *P. saligna* are more closely related to *P. ramulicola* than to an European individual indentified as *P. saligna* (Fig. 11). This may show that they should be regarded as different species separable by morphological characters and life history.

In the present study, we tentatively identified the Japanese representatives as *P. saligna* following Seksjaeva (1989) and Kuroko (1982), but this needs further investigation.

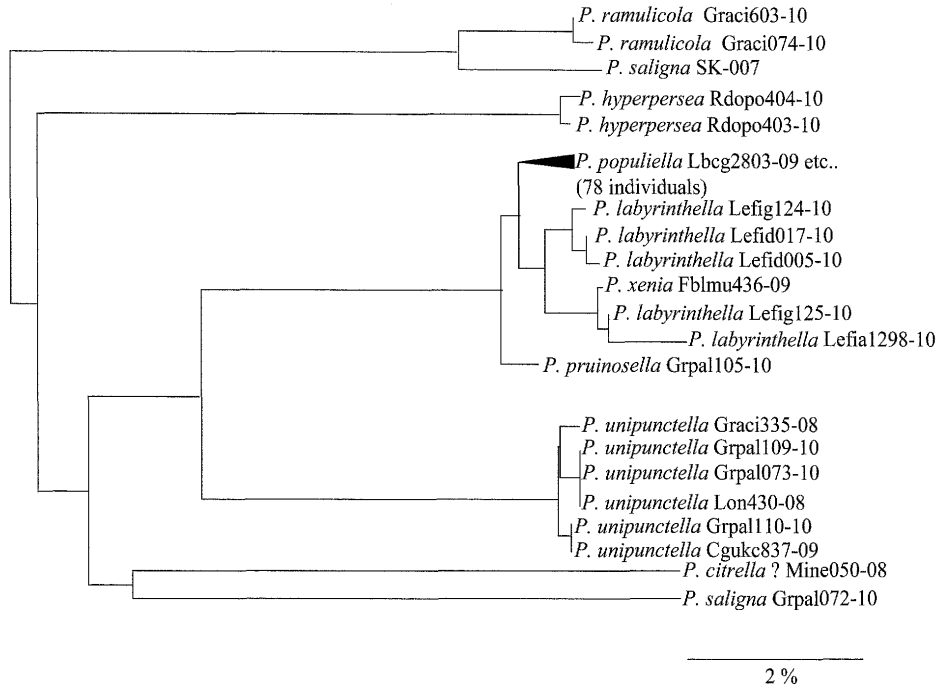

Fig. 11. A tree estimated by neighbor-joining analysis under the Kimura's two-parameter distance model using DNA barcode sequence, retrieved from BOLD identification system (IDS). Branch lengths are proportional to distances. Sequence with ID that started from 'SK-'is obtained from the present study, and the others are from the BOLD database.

Phyllocnistis unipunctella (Stephens, 1834)
(Japanese name "Popura-Kohamoguri")
(Figs 2E, 3C, 5)

Argyromiges unipunctella Stephens, 1834. *Ill. Brit. Ent. Haust.* 4: 260.
Phyllocnistis unipunctella: Seksjaeva, 1997, 429.
Opostega suffusella Zeller, 1847. *Isis*, 12: 894.
Phyllocnistis suffusella: Ermolaev, 1987, 38; Seksjaeva, 1989, 412.

Diagnosis. Forewing lustrous-white with yellowish patch at 1/2, without longitudinal fascia (Table 2, Figs 2E, 3C). Male genitalia with slender valva; valva gradually broadened from 1/4 to apex and curved ventrally (Figs 5A-B). Corpus bursae with a pair of signa with a spine protruding in the center (Fig. 5D).

Description. Wing expanse 5.0-6.0 mm.

Forewing (Figs 2E, 3C); Male genitalia and female genitalia (Fig. 5). See Seksjaeva (1989).

Distribution. Japan: Honshu (Yamagata, Osaka, Hyogo Prefs.); Europe, Central Asia, Russia (Seksjaeva, 1989).

Host plant. *Populus nigra* var. *italica* Du Roi, (Salicaceae) in Japan.

Material examined—7 (4♂1♀2exs)

[Host: *Populus nigra* var. *italica*]: Yamagata Pref.: 3♂, 5. x. 1957 (S. Issiki). Hyogo Pref.: 1♂1♀2exs, Nishinomiya, 8. ix. 1949 em. (S. Issiki).

Biology. Unknown in Japan.

Remarks. The genital structure of this species is similar to that of other species mining poplar and aspen (e. g. *P. labyrinthella* and *P. extrematrix*), but it is distinguishable from them by the characters shown in the diagnosis.

Although Seksjaeva (1997) noted that this species is distributed from Europe to Japan, we have no previous reliable record of it in Japan. The record of this species from Japan by Seksjaeva might be based on the specimen from South Kurile islands (including Etorofu island). Occurrence of this species on mainland Japan is confirmed in this study.

Molecular analysis

Genetic divergence between the new species and P. saligna in Japan. Two genetic clusters, corresponding to *P. gracilistylella* and *P. saligna*, were recognized in thSekse results of neighbor joining analysis on COI sequence data (Fig. 10). Interspecific divergence of the two species is 12% (79 of the 658 bp). On the other hand, intraspecific divergences are very low. Only 2 bp (0.3%) were variable

within each cluster.

Comparison between the two Japanese species and other willow miners available from the database. The BOLD Identification System (IDS) for COI accepts sequence data and returns a species-level identification when one is possible. We could compare DNA barcode data of Japanese *P. saligna* and four European willow-miner species (*P. labyrinthella*, *P. populiella*, *P. xenia*, and *P. unipunctella*). As a result of comparison, the sequence of *P. gracilistylella* was clearly distinguished from all other species by more than 10 % of genetic differences. Japanese representatives of *P. saligna* did not form a clade with a reference specimen identified as the European populations of same species, and the nearest neighbor of the Japanese species was inferred as *P. ramulicola* (Fig. 11). Genetic divergence between Japanese and European *P. saligna* is 12.23 %, while that between Japanese *P. saligna* and *P. ramulicola* is 2.25-2.91 %.

Discussion. To discriminate each species, the pairwise interspecific and intraspecific divergences should be distinctly separated from and should not overlap with each other ("barcoding gap", Meyer and Paulay, 2005). The result reveals that each Japanese species shows very small intraspecific divergence (0.3%), at least between populations in study sites. *Phyllocnistis gracilistylella* is easily distinguished from all other available species by very large (more than 10%) interspecific differences, indicating that this species is separated from all other species in this study. The present result of barcode-based identification also shows that the Japanese population of *P. saligna* is not identical with the European population of the species, suggesting that more detailed study based on materials covering the distribution area is required. This study reveals that the mitochondrial COI sequence is useful to identify *Phyllocnistis* species and to detect cryptic diversity in the group.

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摘　　要

ヤナギ類の葉に潜るコハモグリガ属の1新種の記載，およびヤナギ類に潜る日本産コハモグリガ属の蛹の形態とDNAバーコード領域に基づくヤナギ類潜孔性種の遺伝的比較（小林茂樹・坂本佳子・神保宇嗣・中村彰宏・広渡俊哉）

コハモグリガ亜科Phyllocnistinae（ホソガ科）は，幼虫が葉やまれに茎の表皮にもぐる潜葉性の小蛾類で，成虫は開張4–8 mm，世界におよそ90種が，日本では1属5種が知られる．本亜科には多くの学名未決定種の報告があり，ヤナギ類からは茎に潜るヤナギコハモグリ *Phyllocnistis saligna* (Zeller, 1839) のほかに葉に潜る別種の存在が示唆されていた（平野，私信）．そこで本研究は，ヤナギ類に潜るコハモグリガの形態・生活史の解明に努め，既知のヤナギ類を寄主とする種を含めて形態およびDNAバーコードによる比較をおこなった．

その結果，ネコヤナギなどの葉に潜る1新種，2既知種の計3種をヤナギ類から認めた．ネコヤナギコハモグリ（新種）*Phyllocnistis gracilistylella* sp. nov. とヤナギコハモグリについては幼虫・蛹を観察し，蛹の形態を記載した．新種とヤナギコハモグリの2種は，寄主植物の葉，茎上にそれぞれ同時期・同所的に発生するが，前翅斑紋・雌雄交尾器などの形態形質及び，分子解析でも別種であることが支持された．

ネコヤナギコハモグリは，葉（特に裏側）のみを利用し，寄主範囲も限られていた．一方，ヤナギコハモグリは，若齢幼虫が葉に潜り，その後茎に移り別の葉縁で蛹になり，寄主範囲も *Salix* 属全般にわたった．

1. *Phyllocnistis gracilistylella* Kobayashi, Jinbo & Hirowatari sp. nov. ネコヤナギコハモグリ（新種）(Figs 2A-B, 3A, 4A-D, 6, 8A-D, 9, 10)

開張4.5–6.0 mm. 前翅は銀白色で翅中央に基部から暗色線

が一条走り，1/2 から 2/3 に暗色線に囲まれた黄色帯がある．雄交尾器のバルバは，先端が丸くなる．雌交尾器のシグナは1対でそれぞれ1本の突起を有する．蛹のコクーンカッターは，角状で前上方に突き出る．幼虫は7月から11月にヤナギ類の葉の裏（まれに表側）表皮下に蛇行した線状潜孔を作る．分布：本州（山形，長野，奈良，三重），九州（福岡）．寄主植物：ネコヤナギ，イヌコリヤナギ，カワヤナギ，コゴメヤナギ（ヤナギ科）．

2. *Phyllocnistis saligna* (Zeller, 1839) ヤナギコハモグリ (Figs 2C-D, 3B, 4E-H, 7, 8I-O, 9, 10)

開張6.0–7.0 mm. 前翅は銀白色で翅中央に基部から2/3まで暗色線に囲まれた縦の淡黄色帯が走る．翅形は前種より細長い．雄交尾器のバルバ先端は，腹側に尖り細くなる．雌交尾器のシグナは2個で，それぞれ2–3個の突起を有する．蛹のコクーンカッターは，鉤爪状で，背側に反る．若齢幼虫は，6–11月にヤナギ類の葉に線状潜孔，その後，若枝の表皮下に線状潜孔を作り，葉柄から葉縁の表皮に移り蛹化する．しかし，ヤマナラシ，ネコヤナギでは，葉のみに潜る本種幼虫がみられた．分布：北海道，本州，四国，九州；中国，インド，中央アジア，ロシア，ヨーロッパ．寄主植物：ネコヤナギ，イヌコリヤナギ，オノエヤナギ，カワヤナギ，バッコヤナギ，ヤマヤナギ，ミヤマヤナギ，コゴメヤナギ，タチヤナギ，シダレヤナギ，アカメヤナギ，ヤマナラシ．

3. *Phyllocnistis unipunctella* (Stephens, 1834) ポプラコハモグリ（新称）(Figs 2E, 3C, 5)

開張5.0–6.0 mm. 前翅は銀白色で2/3に黄色帯を有し，中央暗色線を欠く．雄交尾器のバルバは湾曲し，先端はわずかに尖る．大阪府立大学所蔵標本に基づき記録した．日本ではセイヨウハコヤナギに潜るようだが生活史は不明．ロシア極東の文献で南千島から記録されていた．分布：本州（山形，大阪，兵庫），南千島，ロシア，中央アジア，ヨーロッパ．

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